

U.S. DEPARTMENT OF COMMERCE
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PB80-129372

Chemiluminescence of Phagocytic Cells Caused by N-Formylmethionyl Peptides

(U.S.) Health Effects Research Lab, Research Triangle Park, NC

1978

TECHNICAL REPORT DATA		
(Please read Instructions on the reverse before completing)		
1. REPORT NO. EPA-600/J-78-148	2. JOURNAL ARTICLE	3. F. PB80-129372
4. TITLE AND SUBTITLE Chemiluminescence of Phagocytic Cells Caused By N-Formylmethionyl Peptides		5. REPORT DATE
7. AUTHOR(S) Gary E. Hatch, Donald E. Gardner, and Daniel B. Menzel		6. PERFORMING ORGANIZATION CODE
9. PERFORMING ORGANIZATION NAME AND ADDRESS Clinical Studies Division Health Effects Research Laboratory U.S. Environmental Protection Agency Research Triangle Park, NC 27711		8. PERFORMING ORGANIZATION REPORT NO.
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory RTP, NC Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, NC 27711		10. PROGRAM ELEMENT NO. 1AA816
		11. CONTRACT/GRANT NO.
		13. TYPE OF REPORT AND PERIOD COVERED
		14. SPONSORING AGENCY CODE EPA 600/11
15. SUPPLEMENTARY NOTES Published in: J. of Experimental Medicine 147:182-195, 1978		
16. ABSTRACT The microbicidal action of leukocytes is thought to proceed in part through oxygen-dependent reactions. Molecular oxygen can be reduced to superoxide radical anion (O_2^-) which, along with its products, may react directly with the micro-organism, or H_2O_2 and halide may act as substrates for the halogenation of bacteria by myeloperoxidase. Both superoxide and myeloperoxidase-dependent reactions exhibit chemiluminescence in vitro and they appear to be major sources of light emitted from living cells that are in the process of phagocytizing foreign material. Most previous studies of chemiluminescence in leukocytes have employed particulates (zymosan, heat-killed bacteria, and polystyrene butadiene particles), which stimulate phagocytosis as well as O_2 metabolism and in some cases appear to act as substrates in light producing reactions. Interest in the purely oxidative reactions as well as preliminary studies by Allred and Hill led us to the discovery that N-formylmethionyl peptides stimulate chemiluminescence. These unique compounds, which are found in procaryotes, were recently shown to stimulate both chemotaxis and lysosomal enzyme release in leukocytes. In this paper we describe some of the properties of N-formylmethionyl (F-Met) peptide-induced chemiluminescence in human polymorphonuclear leukocytes (PMN) and in PMN and macrophages from guinea pigs and rabbits.		
17. KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Chemiluminescence Phagocytes Peptides	N-Formylmethionyl	06C
18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC RELEASE TO PUBLIC		19. SECURITY CLASS (This Report) UNCLASSIFIED
		20. SECURITY CLASS (This page) UNCLASSIFIED
		21. NO OF PAGES 16
		22. PRICE A02-A01

CHEMILUMINESCENCE OF PHAGOCYtic CELLS CAUSED BY N-FORMYLMETHIONYL PEPTIDES*

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The microbicidal action of leukocytes is thought to proceed in part through oxygen-dependent reactions. Molecular oxygen can be reduced to superoxide radical anion (O_2^-)¹ which, along with its products, may react directly with the micro-organism (1, 2), or H_2O_2 and halide may act as substrates for the halogenation of bacteria by myeloperoxidase (3, 4). Both superoxide and myeloperoxidase-dependent reactions exhibit chemiluminescence in vitro and they appear to be major sources of light emitted from living cells that are in the process of phagocytizing foreign material (5). Most previous studies of chemiluminescence in leukocytes have employed particulates (zymosan, heat-killed bacteria, and polystyrene butadiene particles), which stimulate phagocytosis as well as O_2 metabolism and in some cases appear to act as substrates in light producing reactions (5). Interest in the purely oxidative reactions as well as preliminary studies by Allred and Hill (5a) led us to the discovery that *N*-formylmethionyl peptides stimulate chemiluminescence. These unique compounds, which are found in procaryotes, were recently shown to stimulate both chemotaxis and lysosomal enzyme release in leukocytes (6, 7).

In this paper we describe some of the properties of *N*-formylmethionyl (F-Met) peptide-induced chemiluminescence in human polymorphonuclear leukocytes (PMN) and in PMN and macrophages from guinea pigs and rabbits.

Materials and Methods²

Reagents. Zymosan, xanthine oxidase (grade I), xanthine, horse-radish peroxidase (type 1), superoxide dismutase (bovine blood, 3,000 U/mg), F-Met-Phe, F-Met-Val, F-Met-Ala, Met-Phe, F-Met, and luminol were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium azide was obtained from Fisher Scientific Co., Pittsburg, Pa., and cytochalasin B from Aldrich Chemical Co., Milwaukee, Wis. F-Met-Leu-Phe was a generous gift of Dr. Elliott Schiffmann, National Institutes of Health, Bethesda, Md. Zymosan suspensions were prepared by boiling a solution of

* Supported by EPA Contract no. 68-02-2436.

¹ Abbreviations used in this paper: AM, alveolar macrophage; BSA, bovine serum albumin; F-Met peptides, *N*-formylmethionyl peptides; GBSS, Gey's balanced salt solution; luminol, 5-amino-2,3-dihydro-1,4-phthalazinedione; O_2^- , superoxide radical anion; PM, peritoneal macrophage; PMN, polymorphonuclear leukocyte; SOD, superoxide dismutase.

² **Disclaimer statement.** This report has been reviewed by the Health Effects Research Laboratory, U. S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Gey's balanced salt solution (GBSS, Microbiological Associates, Bethesda, Md.) containing 5 mg/ml of zymosan. The boiled zymosan suspension was then centrifuged, resuspended in GBSS, and kept frozen until use. Superoxide dismutase was dissolved in saline to give a stock solution containing 5 mg/ml.

Isolation of Human Leukocytes. 10 ml of heparinized venous blood obtained by vacutainer (Becton, Dickinson & Co., Rutherford, N. J., heparin sodium 14 U/ml) from two human volunteers was sedimented for 1 h after addition of dextran 70 (Abbott Laboratories, Chemical Div., North Chicago, Ill.). The final concentration of dextran in the blood was 1%. The supernate containing the leukocytes and platelets was layered over lymphocyte separation medium (Bionetics Laboratory Products, Kensington, Md.) and was centrifuged at 400g for 30 min. Mononuclear cells were recovered from the plasma-lymphocyte separation medium interface, and PMN from the bottom of the tube. Erythrocyte contamination of PMN was eliminated by resuspending the cells in 0.83% NH_4Cl , pH 7.2, for 10 min at room temperature. Cellular suspensions were made in GBSS.

Preparation of Alveolar Macrophages and Peritoneal Cells. Male New Zealand white rabbits, 1-2 kg (Pel-Freez Biologicals, Inc., Rogers, Ark.) and Hartley strain guinea pigs, COBS (Cesarean obtained, barrier sustained) 650-800 g (Charles River Breeding Labs, Wilmington, Mass.) were used in these experiments. The technique of Coffin et al. (8) was used to obtain alveolar macrophages (AM). Animals were killed by cervical dislocation, the trachea was exposed, incised, and a catheter inserted. The lungs were then flushed five to six times with saline warmed to 37°C (30 ml for rabbits and 10 ml for guinea pigs). Noninduced peritoneal macrophages (PM) were obtained by pericentesis and lavage with warm saline. Only cellular suspensions containing greater than 98% mononuclear cells were used. Rabbit and guinea pig peritoneal PMN were prepared as described previously (7) by intraperitoneal injection of 60 or 20 ml of saline containing 0.1% glycogen (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) 12-14 h before peritoneal lavage.

Chemiluminescence Measurements. Measurements of chemiluminescence were made with an ATP spectrophotometer (model 3000 SAI Technology Co., San Diego, Calif.). Light intensity in the reaction vial was monitored continuously by use of a strip chart recorder (Fisher Recordall Series 500, Pittsburgh, Pa.), and values of light intensity were recorded in volts. A wide range of sensitivity in the measurement of light could be obtained by changing the sensitivity of the spectrophotometer or the sensitivity of the strip chart recorder. The temperature of the counting chamber of the spectrophotometer was 31°C, therefore, vials containing cells were kept in a waterbath maintained at the same temperature. New plastic scintillation vials (Fisher Scientific Co.) were used throughout, and the chemiluminescence experiments were performed in a partially darkened room. Under these conditions, luminescence readings of the plastic vials were less than 1.0 mV/vial 15 s after placing them in the spectrophotometer. Samples for chemiluminescence measurements were prepared by adding one part of cellular suspension to one part of 1% bovine serum albumin (BSA) in GBSS to form a sample volume of either 1 or 2 ml. Luminol solutions (0.2 mM) were prepared in 1% BSA (Pentex crystallized bovine albumin, Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.) in GBSS by stirring at 4°C until dissolved (6-8 h). The solution was sterilized by micropore filtration and stored in a dark bottle at 4°C. Experiments in which the luminol concentration was varied were performed by making a 0.2 M solution of luminol in dimethyl sulfoxide (Fisher Scientific Co.) and then adding the dimethyl sulfoxide-luminol solution directly to the samples at a dilution of 1:1,000 or 1:2,000. Addition of dimethyl sulfoxide to cell suspensions to final concentrations as high as 0.5% had no effect on subsequent F-Met-Phe-induced chemiluminescence. Dimethyl sulfoxide was also employed to dissolve F-Met peptides when their final concentrations in cells were 0.5 mM or greater. Solutions of luminol in GBSS or GBSS plus BSA prepared by either of the methods described yielded very low levels of light (<3.0 mV/ml). Numerical values for increases in the chemiluminescence of cells were determined by subtracting the basal light intensity (in volts) from the peak light intensity observed after the addition of a stimulant. Most cell preparations exhibited very low basal levels of chemiluminescence (<30 mV/ 10^6 cells with luminol present and <3.0 mV/ 5×10^6 cells in the absence of luminol), and these levels remained low for several hours. The activities of the various peptides, determined by graphical approximation, are reported as the ED₅₀, the concentration giving 50% of the maximal activity. Other tests on the

cells were usually performed at the concentration of F-Met peptide giving 80% of the maximal response (ED80).

Results

Characteristics of the Chemiluminescent Response to F-Met Peptides. Chemiluminescence induced by F-Met peptides was different from that produced by the commonly used particulate stimulant, zymosan, in that maximal levels of light intensity were reached sooner (Fig. 1). The major burst of light occurred within 2 min after addition of F-Met-Phe while the maximum zymosan-induced chemiluminescence occurred after 8 min. F-Met peptides other than F-Met-Phe also exhibited a similar time-course. After an initial burst of light, F-Met peptide-induced chemiluminescence appeared to decrease exponentially with a half-life of 1-2 min, but light emissions remained above basal levels for at least 15 min. A chemiluminescent response to F-Met-Phe was also observed with rabbit and guinea pig PMN as well as guinea pig AM and PM. The time-courses of response in these cells were similar to that observed in the human PMN.

Luminol Enhancement of Chemiluminescence. Luminol increased the intensity of F-Met peptide and zymosan-induced chemiluminescence, while the time courses of these responses remained the same as they were in the absence of luminol. Fig. 2 shows that the addition of 10^{-7} M luminol resulted in approximately a 10-fold enhancement of existing chemiluminescence. Increasing the luminol concentration to 10^{-6} M also resulted in a 10-fold enhancement, but at concentrations of 10^{-5} and 10^{-4} M only a 5-fold enhancement over the previous lower concentration was seen. Higher concentrations of luminol were not possible because of its limited solubility. Both F-Met-Phe and zymosan-induced chemiluminescence appeared to be enhanced similarly by luminol. A luminol concentration of 10^{-4} M was arbitrarily chosen for the remainder of the experiments reported here because it was near the maximal enhancement of

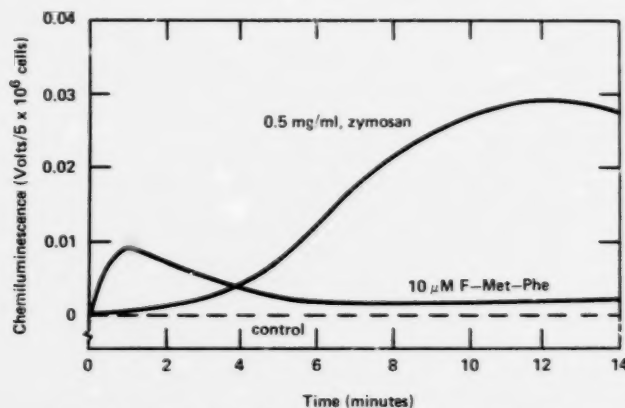


FIG. 1. Time-course of the chemiluminescent response of human PMN induced by $10 \mu\text{M}$ F-Met-Phe and 0.5 mg/ml zymosan. Each 2 ml test solution contained 5×10^6 PMN and 0.5% BSA in GRSS. Data were obtained by tracing the record of chemiluminescent intensity.

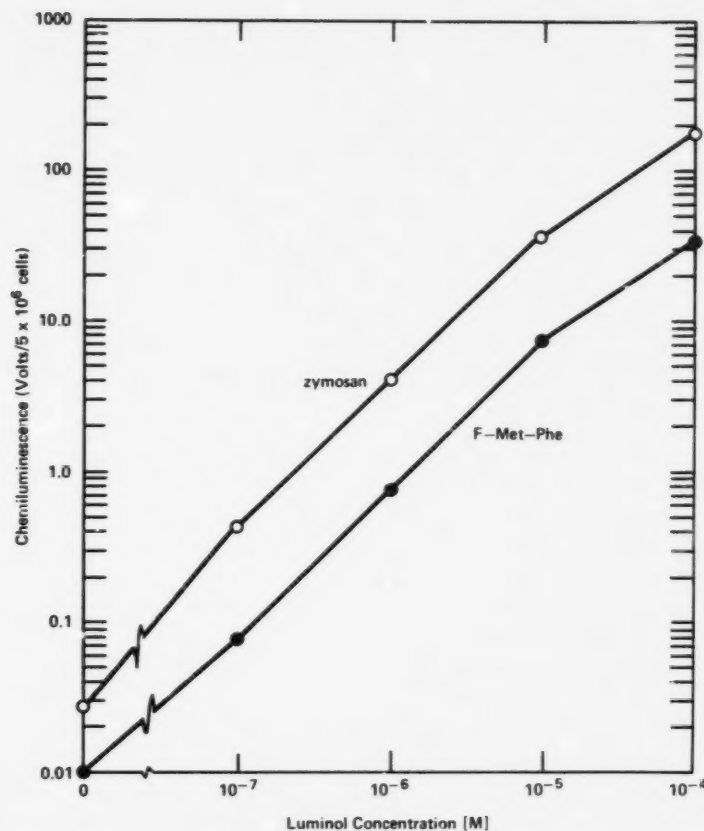


FIG. 2. The effect of luminol concentration on the chemiluminescent response of human PMN stimulated by F-Met-Phe and zymosan. Human PMN (5×10^6 cells/2 ml sample) were suspended in GBSS containing 0.5% BSA. The concentrations of F-Met-Phe and zymosan were 1 μ M and 0.5%, respectively.

light intensity observed. At this concentration, luminol increased light output 3,000–6,000-fold over that seen in its absence.

Cell-Dependence of F-Met-Phe-Induced Chemiluminescence. No light production was seen when 10 μ M F-Met-Phe was added to 10^{-4} M luminol in 0.5% BSA with no cells present, or to cells that were disrupted by sonication or killed by heat-treatment (100°C, 3 min). The concentration of cells in the counting vials also affected the luminescent response to F-Met-Phe (Fig. 3). When the concentration of cells was increased from 0.1 to 10×10^6 cells/ml, the chemiluminescent response to an ED80 dose of F-Met-Phe was also increased proportionately. In addition, cells previously exposed to F-Met-Phe were less responsive for at least 1 h to a second challenge of the peptide. Larger initial doses gave greater deactivation. Previous addition of F-Met-Phe also caused deactivation when cells attached to glass vials were washed free of the initial peptide solution and then rechallenged.

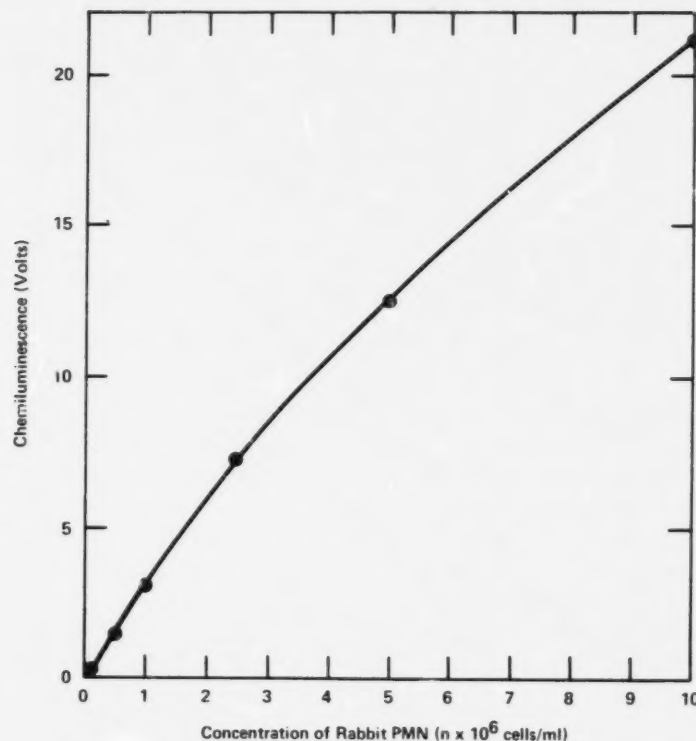


FIG. 3. Dependence of F-Met-Phe-induced chemiluminescence on cell concentration. Each value represents the mean of three determinations. Standard deviations are within the size of each point. Each 2 ml test sample contained the given concentration of rabbit peritoneal PMN, 0.5% BSA, and 0.1 mM luminol. The ED80 concentration of F-Met-Phe for these cells ($2 \mu\text{M}$) was added to each vial, and the peak chemiluminescence recorded.

Effect of F-Met-Phe in Different Cell Types. Chemiluminescent responses in the presence of luminol were highly reproducible within a given batch of cells and easily measured even when only a small number of cells was available. Different cell types were compared for their ability to respond to F-Met peptides in the presence of luminol. Table I shows that the maximal chemiluminescent response attainable with the model peptide, F-Met-Phe, was different in different cell types. Human PMN were more active by an order of magnitude than other cells. Guinea pig AM, PM, PMN, and rabbit PMN had intermediate chemiluminescent activity. Rabbit AM did not appear to respond to the peptides although they did respond to unopsonized zymosan ($366 \text{ mV}/10^6 \text{ AM}$ for 0.5 mg/ml zymosan). A small but measurable F-Met-Phe-induced chemiluminescent response was seen in rabbit PM, however, these cells did not respond as well as the AM to unopsonized zymosan ($6 \text{ mV}/10^6$ cells). The human mononuclear cell fraction always contained at least 10% PMN. Because of the high chemiluminescent activity of human PMN we were unable to determine whether the monocytes or lymphocytes of human blood

TABLE I
Maximal Chemiluminescent Response to F-Met-Phe in Different Cell Types

Cell type	Maximal F-Met-Phe-induced chemiluminescence (V/10 ⁶ cells)*
Human PMN	120.0 ± 76.0 (5)
Guinea pig AM	13.0 ± 2.9 (3)
Guinea pig peritoneal PMN	7.2 ± 2.4 (5)
Rabbit peritoneal PMN	7.9 ± 0.7 (3)
Rabbit AM	0.001 ± 0.001 (3)
Rabbit PM	0.002 ± 0.001 (4)

* Each value represents the mean ± SD of the maximum increase in chemiluminescence seen in a dose-response curve to F-Met-Phe. The number of cell preparations is in parentheses. All measurements were made on 1-ml samples, each of which contained $0.3\text{--}0.5 \times 10^6$ cells, 0.5% BSA, and 0.1 mM luminol.

can be stimulated by the F-Met peptides to give a chemiluminescent response.

Structure Activity Relationships of Peptides in the Chemiluminescent Assay. The dose-response behavior of F-Met peptides were examined in human PMN, in glycogen-induced PMN and noninduced AM and PM of rabbits and guinea pigs. A representative experiment in Fig. 4 shows that when the increase in chemiluminescence was plotted against the logarithm of the molar concentration of peptide, a sigmoidal-shaped dose-response curve was obtained. As peptide concentrations were increased above those which produced a maximal response, the chemiluminescence was often decreased. Maximal chemiluminescent responses in a given batch of cells were the same for F-Met-Leu-Phe, F-Met-Phe, and F-Met-Val; however, F-Met-Ala consistently yielded a lower maximal response than the other peptides. No chemiluminescent response could be detected after addition of millimolar concentrations of F-Met, Phe, or Met-Phe in any of these experiments. Table II presents data from several experiments similar to that shown in Fig. 4. The relative potencies of the formylated peptides in inducing chemiluminescence was the same in all cell types examined, e.g., F-Met-Leu-Phe $\gg$ F-Met-Phe > F-Met-Val > F-Met-Ala. Similar results were obtained in experiments performed without luminol present. For example, ED50 values for the F-Met peptide induced chemiluminescence of guinea pig PMN in the absence of luminol were: F-Met-Leu-Phe, 2.0 nM; F-Met-Phe, 0.45 μ M; F-Met-Val, 21.0 μ M; F-Met-Ala, 1.1 mM. These ED50 values compare favorably with experiments performed with luminol present (Table II). Some differences between cell types could be noted in the absolute potencies of the peptides, for example, ED50 values for all of the peptides, except F-Met-Ala, were about 10-fold higher in human PMN than in the other cell types.

The Effect of Superoxide Dismutase on F-Met Peptide-Induced Chemiluminescence. Fig. 5 shows that addition of low concentrations of SOD to suspensions of guinea pig PM and PMN resulted in a dose-related inhibition of F-Met-Phe-induced chemiluminescence. Complete inhibition of chemiluminescence was achieved by 10 μ g/ml of superoxide dismutase in these cells, in human

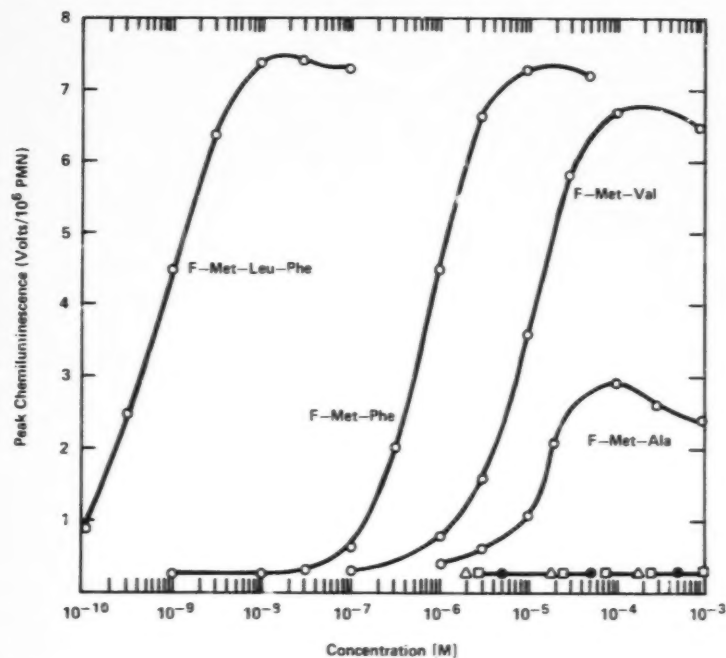


FIG. 4. The effect of peptide and amino acid structure and concentration on ability to induce chemiluminescence in rabbit PMN. All measurements were made on 1-ml samples containing 0.5×10^6 cells, 0.5% BSA, and 0.1 mM luminol in GBSS. Effects of Met-Phe, Δ ; F-Met, $\square$; Phe, $\bullet$; and formylated dipeptides as labeled, $\circ$; are represented.

TABLE II
Comparison of ED50 Values of Four F-Met Peptides in Inducing Chemiluminescence in Different Cell Types*

Cell type	F-Met-Leu-Phe	F-Met-Phe	F-Met-Val	F-Met-Ala
	nM	μ M	μ M	mM
Human PMN	63.0 ± 28.0	1.9 ± 0.17	16.0 ± 3.1	2.6 ± 1.7
Guinea pig PMN	5.3 ± 1.1	0.18 ± 0.02	2.5 ± 0.6	1.3 ± 0.2
Guinea pig AM	6.0 ± 2.8	0.20 ± 0.007	3.5 ± 0.7	2.0 ± 1.2
Guinea pig PM	8.7 ± 2.1	0.20 ± 0.006	5.0 ± 1.2	5.5 ± 2.4
Rabbit PMN	0.95 ± 0.3	0.64 ± 0.2	16.0 ± 4.0	5.9 ± 3.9

* Concentrations of the F-Met peptides which produced a $1/2$ maximal chemiluminescent response (ED50) are shown. Each concentration value represents the mean $\pm$ SD of the ED50 calculated from three separate cell preparations. All measurements were made on 1-ml samples containing $0.3\text{--}0.5 \times 10^6$ cells, 0.1 mM luminol, and 0.5% BSA in GBSS.

PMN, and in PM and AM from guinea pigs. The luminol-enhanced chemiluminescent response was also completely inhibited by this concentration of SOD.

The Effect of Cytochalasin B and Azide on F-Met Peptide-Induced Chemiluminescence. The role of lysosomal enzyme release in F-Met-Phe-induced chemiluminescence was examined in guinea pig PMN and PM (Table III).

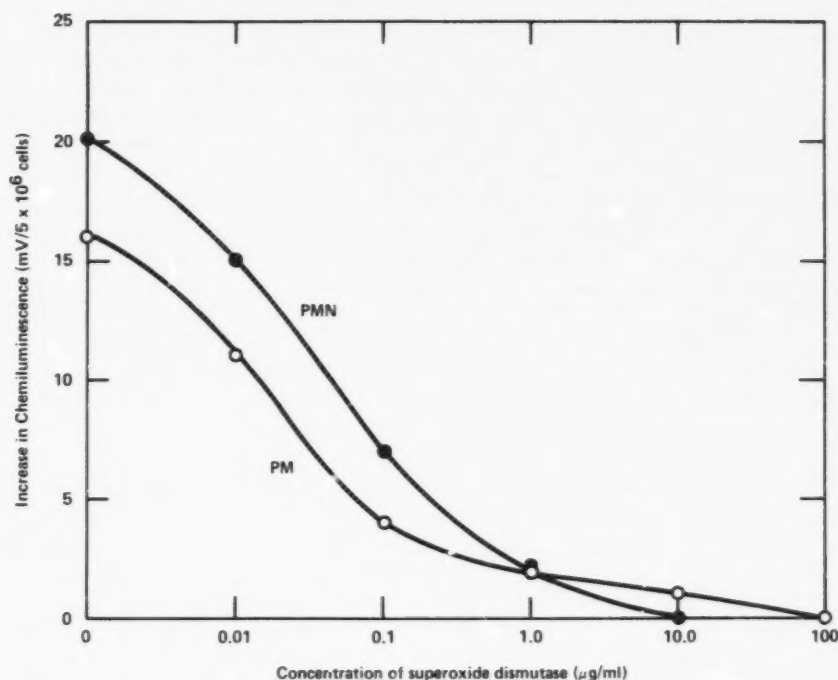


FIG. 5. Inhibition of the F-Met-Phe-induced chemiluminescent response by SOD. Each 1 ml sample contained 5×10^6 cells, 0.5% BSA in GBSS, and the ED80 concentration of F-Met-Phe for that batch of cells. Inhibition by SOD is shown for guinea pig peritoneal PMN (●) and guinea pig PM (○). Mean values are plotted ($n = 1-3$ per point).

TABLE III
The Effect of Cytochalasin B and Azide on F-Met-Phe-Induced Chemiluminescence*

ED80 Dose of F-Met-Phe (control)	Increase in chemiluminescence (percent of control $\pm$ SE)	
	Peritoneal macro- phage	Polymorphonuclear leukocyte
	100%	100%
" + 1 μ M Cytochalasin B	81.6 $\pm$ 4.40%	336 $\pm$ 109%
" + 0.1 mM NaN ₃	89.4 $\pm$ 10.9%	69.5 $\pm$ 5.68%
" 1 μ M Cytochalasin B + 0.1 mM NaN ₃	78.9 $\pm$ 6.5%	83.7 $\pm$ 7.34%

* All measurements were made on 1-ml samples containing $0.3-0.5 \times 10^6$ guinea pig cells, 0.1 mM luminol, and 0.5% BSA in GBSS. Each percentage value was calculated from the increases in chemiluminescence seen in three to four cell preparations from different animals. The ED80 concentration of F-Met-Phe was approximated graphically for each cell preparation before the experiment. All experiments were performed by preincubating cells for 5 min with NaN₃ and/or cytochalasin B before addition of F-Met-Phe.

Cytochalasin B, which causes extracellular release of lysosomal enzymes in cells stimulated by the F-Met peptides (7) caused a threefold enhancement of chemiluminescence in PMN, and a small inhibition of chemiluminescence in PM (Table III). The enhancement of F-Met-Phe-induced chemiluminescence by cytochalasin B was completely abolished by addition of the peroxidase inhibitor, sodium azide. Azide (0.1 mM) by itself did not appear to have a significant effect in macrophages, while it generally inhibited to some degree the response in PMN. In other experiments, human PMN also showed an enhanced chemiluminescent response to 1 μ M cytochalasin B (275% over control) that was inhibitable by azide. Guinea pig AM gave results similar to those seen with the PM. Therefore, F-Met-Phe-induced chemiluminescence in PMN showed an azide-inhibitable enhancement in the presence of cytochalasin B, while macrophages showed no enhancement. This effect was seen both in the presence and absence of luminol.

Discussion

PMN isolated from human blood, as well as PMN and macrophages obtained from guinea pigs and rabbits chemiluminesce when stimulated by certain *N*-formylmethionyl peptides. These peptides, which are believed to be present in bacteria but not mammalian cells, have been shown previously to be recognized at low concentrations by phagocytes. They are capable of initiating both the secretion of lysosomal enzymes and directed cell migration (9, 10). Results presented here suggest that yet another cellular activity might be initiated by these peptides, e.g., the production of highly reactive molecules of possible importance in microbial killing.

The cellular nature of F-Met-Phe-induced chemiluminescence was indicated by its dependence on cell concentration, and its inhibition by cell disruption, heat inactivation, or previous maximal stimulation of the cell by the peptide. Luminol, a chemiluminescent indicator of oxidizing species, enhanced in a dose-related manner the intensity of the chemiluminescent response caused by both zymosan and F-Met-Phe (Fig. 2). No difference was seen between the time courses of the luminol-enhanced and the luminol-independent chemiluminescent responses. This effect was in agreement with previous studies with zymosan (11). The use of luminol to enhance chemiluminescence made the study of the effects of the peptides more facile in both PMN and noninduced macrophages.

Comparison of different cell types in their ability to respond to F-Met-Phe in the presence of luminol showed marked differences between species (Table I). The maximal chemiluminescence attainable in a dose-response curve to F-Met-Phe ranged from near zero in rabbit macrophages to very high levels (120 V/10⁶ cells) in human PMN. Glycogen-induced rabbit and guinea pig peritoneal PMN gave chemiluminescent responses of intermediate intensity, as did non-induced macrophages of the guinea pig. Rabbit AM gave no chemiluminescent response to the peptides, while they did respond to zymosan, suggesting perhaps that these cells lack the receptor for F-Met peptides.

Dose-response studies with different F-Met peptides showed that molecular structure had large effects on the potency of the peptides in stimulating

chemiluminescence. For example, Met-Phe, F-Met, and Phe had no measurable activity even at millimolar concentrations, while F-Met-Phe was active in the micromolar range (Fig. 5). The same relative potency was seen for the peptides whether human, rabbit, or guinea pig PMN or guinea pig AM or PM were tested (Table I). Relative potencies of the four active peptides, as indicated by their ED₅₀ values was in all cases F-Met-Leu-Phe $\gg$ F-Met-Phe $>$ F-Met-Val $>$ F-Met-Ala. This relationship was seen both in the presence and absence of luminol. Average ED₅₀ values of the peptides in inducing chemiluminescence (Table II) are similar to ED₅₀ values of the peptides reported previously for the induction of chemotaxis and lysosomal enzyme release. For example, Showell et al. (7) reported the ED₅₀ values of F-Met-Leu-Phe and F-Met-Phe as 0.24 nM and 1.5 μ M, respectively, for the induction of lysosomal enzyme release in rabbit PMN. Average ED₅₀ values for the induction of chemiluminescence in the same type of cells (Table II) were 0.95 nM and 0.64 μ M. Relative activities of F-Met-Phe, F-Met-Val, and F-Met-Ala reported previously by Schiffmann et al. (6) in inducing chemotaxis in rabbit PMN also appear to be similar to the relative activities of these peptides in inducing chemiluminescence. The only discrepancies that may exist are that the earlier studies reported chemotactic and lysosomal enzyme releasing activity for high concentrations (1-10 mM) of F-Met, while we were unable to see a chemiluminescent response to this agent. Also, dose-response curves of the peptides in inducing the former responses showed the same maxima for all peptides, while dose-response curves in the chemiluminescent response had an exception, e.g. F-Met-Ala always produced a lower maximal response than the other peptides. The reason for this effect is not yet apparent.

Further investigations were directed at understanding the nature of the chemiluminescent molecules produced by the cells in response to the F-Met peptides. Inhibition of the F-Met peptide-induced chemiluminescent response by SOD (Fig. 5) suggests the involvement of O₂⁻ or O₂⁻-derived compounds. SOD inhibition was complete in all cells examined (no luminol present) when a concentration of 10 μ g/ml was used. Using a variety of other cellular stimulants, previous investigators have also observed an inhibition of chemiluminescence by SOD (9, 10). Beall et al. (12) showed recently that human alveolar macrophages gave a chemiluminescent response to heat-killed bacteria and to phorbol myristate acetate that was completely inhibited by 100 μ g/ml SOD. Zymosan-induced chemiluminescence in human PMN, however, was only partially (55%) inhibited by SOD (94 μ g/ml) in studies by Rosen and Klebanoff (5). They suggest that the zymosan-induced chemiluminescence in human PMN is due to both azide-inhibitable peroxidases and O₂⁻-dependent reactions. Since our studies show complete inhibition of F-Met peptide-induced chemiluminescence by SOD in all cells examined including human PMN, F-Met peptides and zymosan may differ in their ability to release lysosomal myeloperoxidase. Differences may also exist in the nature of the oxidizable substrates which participate in the chemiluminescent reactions. Zymosan was shown by Rosen and Klebanoff to be a substrate in chemiluminescent reactions of myeloperoxidase and xanthine oxidase. It seems unlikely that the F-Met peptides are substrates in chemiluminescent reactions because of their low

concentrations. Also, repeated additions of the peptides do not lead to repeated enhancement of light production, as might be expected if they were substrates.

The small amount or lack of inhibition by azide of F-Met-Phe-induced chemiluminescence suggested that myeloperoxidase reactions are not major sources of light under the conditions used in these experiments. Since myeloperoxidase is present in the dense granules of PMN (13), its release into the medium surrounding the cells might be an expected prerequisite for its involvement in a chemiluminescent response. Support of this idea was found in the effects of cytochalasin B. Cytochalasin B, which is known to cause external release of lysosomal enzymes in PMN stimulated with F-Met peptides (7), also enhanced F-Met-Phe-induced chemiluminescence in PMN. Macrophages, however, showed no such enhancement, in agreement with the reported lack of granule-associated myeloperoxidase in these cells (14, 15). The enhancement of chemiluminescence caused by cytochalasin B was inhibited by the peroxidase inhibitor, sodium azide, also supporting the conclusion that the increased chemiluminescence was due to myeloperoxidase release.

All of the experiments reported here could be performed either in the presence or absence of luminol with similar results. Luminol made possible a greater number of experiments in a given batch of cells. Unfortunately, the chemical nature of both native and luminol-enhanced chemiluminescence have not been worked out in enough detail to provide an adequate comparison of the two. Our experiments suggest that luminol scavenges the same oxidizing species as causes native chemiluminescence. Both O_2^- -forming (e.g., xanthine-xanthine oxidase) and peroxidase-catalyzed reactions yield native chemiluminescence (5, 16, 17) and oxidize luminol (18, 19). Both types of reaction have counterparts in leukocyte metabolism. Metal ions also catalyze luminol oxidation, but only in alkaline solutions (18, 20). Inhibition of luminol chemiluminescence by SOD apparently does not prove that O_2^- is responsible for its oxidation. Hodgson and Fridovich (18) have suggested that O_2^- is formed during aerobic luminol oxidation initiated by oxidants other than O_2^- . Therefore, SOD appears to inhibit luminol luminescence regardless of the nature of the oxidant which initiates its oxidation (21). Zymosan appears to be a substrate in chemiluminescent reactions of leukocytes, therefore it is possible that luminol is also a substrate for oxidizing chemiluminescent reactions. The use of model cellular stimulants as well as model substrates may provide new avenues for elucidating the microbicidal mechanisms of phagocytic cells.

The observed similarities in the structure-activity relationships for chemiluminescence, lysosomal enzyme release, and chemotaxis raises the question of whether one of the processes is a direct result of the other. For example, if chemiluminescent substances were released with lysosomal enzymes it would account for the similar structure-activity relationships of the two processes. Recent reports indicate, however, that O_2^- and H_2O_2 are not released with the lysosomal enzymes (22, 23) and therefore it would be expected that chemiluminescence caused by direct reactions of these agents would occur independently of enzyme release. Another explanation might be that the granule-associated myeloperoxidase reacts with H_2O_2 extracellularly or in the phagocytic vacuoles, and thus accounts for the observed structure-activity relationship of F-Met

peptide-induced chemiluminescence. This seems unlikely because sodium azide, an inhibitor of myeloperoxidase, had little inhibitory effect on chemiluminescence caused by F-Met peptides unless lysosomal enzyme release was enhanced by cytochalasin B. Also, macrophages did not exhibit an azide-inhibitable chemiluminescent response even when cytochalasin B was present, indicating that peroxidases are not involved in the chemiluminescence of these cells. Thus, it seems likely that the F-Met peptides trigger the O_2^- -forming machinery of the cell in addition to activating lysosomal enzyme secretion. If this is so, the apparent similarities in the structure-activity relationships of F-Met peptide-induced chemiluminescence, chemotaxis and lysosomal enzyme release would indicate that these processes result from activation of a single receptor mechanism.

Summary

N-formylmethionyl (F-Met) peptides, when added alone to macrophages or polymorphonuclear leukocytes (PMN), were found to induce a chemiluminescent response of shorter duration than that produced by the commonly employed particulate stimulant, zymosan. The cellular nature of F-Met peptide-induced chemiluminescence was indicated by its dependence on cell concentration, and by its inhibition by cell disruption, heat inactivation, or previous maximal stimulation by the peptides.

Comparison of PMN and macrophages from different species showed that the maximal chemiluminescent response seen in the dose-response curve of F-Met-Phe was different in different cell types. Chemiluminescence reached highest values in human PMN, it was intermediate in guinea pig macrophages and PMN, and in rabbit PMN; but it was nonexistent in rabbit alveolar macrophages and very low in rabbit peritoneal macrophages. A definite relationship was observed between peptide structure and chemiluminescent activity. Met-Phe, F-Met and Phe were inactive even at millimolar concentrations, while F-Met-Phe caused chemiluminescence at micromolar concentrations. Four active peptides were tested in guinea pig, rabbit, and human PMN, and in guinea pig alveolar and peritoneal macrophages. The relative activity of these peptides was the same in all cells studied, e.g. F-Met-Leu-Phe $\gg$ F-Met-Phe $>$ F-Met-Val $>$ F-Met-Ala. The values of ED50 for each peptide were also comparable to previously reported ED50 values of these peptides in inducing lysosomal enzyme release. These results were seen both in the presence and absence of the chemiluminescent oxidant indicator, luminol.

Low concentrations of superoxide dismutase (10 μ g/ml) completely inhibited chemiluminescence caused by the F-Met peptides, suggesting the involvement of O_2^- or O_2^- -derived compounds in this response. Sodium azide, an inhibitor of peroxidase reactions, had either no effect or a slight inhibitory effect on chemiluminescence. However, when the extracellular release of lysosomal enzymes was induced by cytochalasin B, an azide-inhibitable enhancement of chemiluminescence was seen in PMN, but not in macrophages. This effect appears to be correlated with the presence of granule-associated myeloperoxidase. Although azide-inhibitable peroxidases could be a potential source of light, they did not appear to be a significant contributor in these experiments.

Based on these results and on those of previous investigators, we postulate that the F-Met-peptides stimulate O_2^- production in addition to stimulating lysosomal enzyme release and chemotaxis. The similar structure-activity relationship which appears to exist for these processes may indicate that they are all initiated by a single receptor mechanism. Since F-Met peptides are formed in bacteria it is likely that their actions represent an important physiologic response.

The expert technical assistance of Mr. David N. Drechsel is gratefully acknowledged.

Received for publication 21 September 1977.

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